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These experiments investigate a mouse model for the biosynthesis of the human adrenal androgens (dehydroepiandrosterone, DHEA, and its sulfate, DHEAS) and the role of these steroids in human breast cancer growth. An androgen-dependent human breast cancer model was established in immunodeficient (*scid*) mice. Zona reticularis cells in the human adrenal cortex are responsible for adrenal androgen biosynthesis because of the suppressed expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in these cells. A protein present in the non-DHEA-secreting zones of the cortex and absent from the zona reticularis which binds to a regulatory region of the type II 3β-HSD gene was partially purified. Human adrenocortical cells were transplanted into *scid* mice and were shown to replace the animals' own adrenal function. Although zona reticularis cells were transplanted, DHEAS was not detected in mouse plasma. As an alternative to the use of human zona reticularis cells, clonal bovine adrenocortical cells were shown to be capable of forming tissue in *scid* mice that replaces the animals' adrenal glands. This was shown both with normal clonal cells and with cells genetically modified by the insertion of marker genes. The ability to genetically modify the cells provides a means to test whether suppression of 3β-HSD by an antisense strategy can create a tissue with a very high rate of DHEA biosynthesis in the mouse transplant model.

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Peter Hung 9/29/98

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Introduction

Unlike human cancers which are clearly correlated with readily identifiable aspects of lifestyle, for example lung cancer and smoking history, breast cancer does not appear to be dependent on exposure to specific environmental carcinogens. Rather, the major risk factor for breast cancer in women appears to be the lifetime exposure of breast tissue to circulating and local natural estrogens and androgens. Estrogens of various types can act both as co-initiators of carcinogenesis (together with other agents -- possibly universally present carcinogens) and as growth factors for breast cancer tissue, particularly in a subset of tumors which are strongly dependent on estrogen for growth. Estrogens to which the breast tissue is exposed are of both ovarian and extra-ovarian origin. In both men and women, the major source of tissue estrogens are circulating androgens and androgen precursors which are locally converted via aromatase to an active estrogen. The major circulating androgen precursor is dehydroepiandrosterone (DHEA) and its sulfate, DHEAS. These androgen precursors originate overwhelmingly from the adrenal cortex in women rather than from the ovary. Surprisingly, it has also recently been shown that even testosterone in the ovarian follicle itself can be formed from circulating DHEAS of adrenocortical origin. For these reasons, it is clear that production of DHEAS and other androgens by the adrenal cortex forms a major lifetime risk factor for breast cancer development in women.

From the early part of this century it was known that adrenalectomy improves survival in a substantial fraction of women with breast cancer. Although no longer performed, because of an unacceptable incidence of complications, adrenalectomy was effective because it removed the source of adrenal androgens and deprived the breast cancer of its major source of estrogens.

The aim of the studies here is to provide basic information on the regulation of androgen precursor synthesis by the human adrenal cortex and to test the effects of adrenal androgen synthesis on human breast cancer growth in a mouse model. The immunodeficient *scid* mouse is used both as host for functional human adrenal organoids as a source of androgens and has human breast cancer cells implanted as a target tissue.

Uses of the information obtained on human adrenocortical DHEAS production are the identification of hormonal and molecular factors that set the adrenal androgen production level. This information may more precisely define the risk factors of adolescent and postadolescent women for higher peak levels of DHEAS and consequent increased exposure of the breast tissue to estrogens. The better characterization of the factors that regulate adrenal androgen synthesis, currently very poorly defined both molecularly and physiologically, would enable appropriate diagnosis and interventions in high-risk women and may provide other avenues of rational treatment in estrogen-responsive breast cancer.

Body

The aims of the funded grant are to set up a mouse model for human adrenal androgen production by the adrenal cortex and the effects of these androgens on the growth of human breast cancer.

The specific tasks were as follows:

1. Further develop the human adrenal organoid/*scid* (severe combined immunodeficiency) mouse model for investigation of the regulation and effects of human adrenal androgens.

2. Assess the influence of circulating adrenal androgens on human estrogen-responsive human breast cancer cell growth.

3. Investigate the molecular biology of adrenal androgen regulation, focusing on the key enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD).

4. Assess physiological influences on adrenal androgen production in the human adrenal organoid/*scid* mouse model.

5. Identify transcription factors which regulate the human type II 3β-HSD gene and test their effects on adrenal androgen synthesis in the human organoid/*scid* mouse model.

The accomplishments of this grant over the past four-year period have been as follows:

1. An immunodeficient mouse/xenotransplant model has been set up in which human adrenocortical cells are grown as a functional tissue in the *scid* mouse. The human adrenal cells, now vascularized and forming a functional tissue structure, replace the animal's own adrenal glands and secrete steroids which maintain its health and life. This is the first time that a mouse endocrine organ has been replaced entirely in its function by transplanted human cells. This humanized mouse model can be used to study aspects of adrenal physiology, cell biology and biochemistry, which would be impossible in intact human subjects. Our specific aim with respect to this grant was to establish whether these transplanted tissues secrete the adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS).

During the period of this grant we made the discovery that DHEA and DHEAS are secreted exclusively from the zona reticularis cells of the adrenal cortex. These cells form the innermost zone in the adult human adrenal cortex. On the other hand, the zona fasciculata cells of the human adrenal cortex, which form the middle zone, secrete the glucocorticoid cortisol and do not secrete adrenal androgens. We prepared these two cell types separately from the human adrenal cortex and transplanted them into *scid* mice. Both cell types formed functional tissues, but, in both cases, the tissues secreted cortisol without detectable DHEAS in the mouse plasma. This could indicate that the cells lost their ability to make DHEA. However, there are other possibilities to consider. The first possibility is that the cells re-differentiate after transplantation and that they tend to differentiate into zona fasciculata cells, rather than zona reticularis cells. This indicates a need for more understanding of the way in which the zonation of the adrenal cortex is set up and how this differentiation is regulated at the molecular level. This aspect of the research is addressed in a further section. The second possibility is that in mice DHEAS may not achieve the high circulating levels that it does in humans, even if DHEA is entering the circulation. We did experiments in which DHEA was administered to mice in their drinking water. Plasma DHEAS levels did not rise to detectable levels. Thus, there are extra-adrenal differences between mouse and human physiology which will need to be addressed before the mouse can be used as a totally reliable model of human adrenal physiology. However, the experiments performed under this grant provide the basis for future studies. Human adrenal tissue has been established in mice, and the data thus far indicate a need to understand the basis of the functional differentiation of the cells into the zones of the human adrenal cortex. Even if DHEAS levels do not rise in mouse plasma, there may nevertheless be androgenic effects of DHEA secreted by the transplanted cells on target mouse tissues. In experiments done by others, in which DHEA was administered to rodents, androgenic effects on target tissues such as the prostate were clearly seen.

Other experiments have been designed to investigate if we zonation can be regulated in the transplanted cells by altering the protocol for transplantation. We developed polymer foams formed by modification of poly(L-lactic acid) to enable covalent binding of biopolymers. These foams can be used for high-density three-dimensional culture of adrenocortical cells, and we thought it was possible that they might support a larger scale transplant structure in vivo. However, they did not support adrenocortical cell growth in the transplant model. Other ways to control the zonation of the transplant tissue are needed, as described below.

2. The transplantation of genetically modified adrenocortical cells in the mouse model.

A critical need has been to be able to genetically modify the cells prior to transplantation. This was explicitly proposed in the grant application, but not all of the procedures necessary to realize this aim were in place at that time; many of them have been developed during the period this grant. At this point, we are a much better position to directly test the results of genetic modification on the function of human adrenal cells in the mouse model. The following progress has been made:

First, we formed clonal tissue from normal (not genetically modified) clonal bovine adrenocortical cells. Attempts to form tissue from clonal human adrenocortical cells have been unsuccessful. Nevertheless, we believe that these difficulties are predominantly technical in nature and we expect that we will be able to perform the same kinds of experiments on human adrenocortical cells that we have performed on bovine cells. The ability to form tissue from clonal cells is a necessary step for the production of tissue from genetically modified cells, because the selection of transfected cells in culture which is required for genetic modification produces clonal populations of cells. Therefore we reasoned that the production of tissue from normal clonal cells would be a pre-requisite for the production of tissue from genetically modified clonal cells.

For bovine cells, we showed that xenotransplanted adrenocortical tissue of clonal origin can be formed in *scid* mice by using techniques of cell transplantation. We studied in detail a single clone of bovine adrenocortical cells, but 5 of 20 other randomly selected clones also formed tissue. Most adrenalectomized animals bearing transplanted cells survived indefinitely, demonstrating that the cells restored the animals' capacity to survive in the absence of sodium supplementation. Formation of well-vascularized tissue at the site of transplantation was associated with stable levels of cortisol in the blood, replacing the mouse glucocorticoid (corticosterone). Ultrastructurally, the cultured cells before transplantation had characteristics of rapidly growing cells, but tissue formed *in vivo* showed features associated with active steroidogenesis. These experiments show that an endocrine tissue, functionally replacing the corresponding tissue of the host animal, can be derived from a single normal somatic cell.

Moreover, for bovine adrenal cells, we have produced genetically modified tissue, which is functional and replaces the normal adrenal function of the animals. This is the first time that a xenotransplanted genetically modified tissue has been used to replace the essential function of one of an animal's organs. The genes that were used to modify the cells so far are essentially "marker" genes. We do not believe that they have had functional significance, although some caviats are necessary with respect to this statement. The genes which have been introduced are the genes for the green fluorescent protein (in the enhanced version as marketed by Clontech); second, the *neo* gene, which confers G418 resistance and is commonly used for cell selection; and third, the SV40 T antigen gene. Interestingly, we have found that SV40 T antigen so far is necessary for the production of the genetically modified tissue, yet it does not appear to substantially affect the function of the tissue. We previously showed some years ago that SV40 T antigen increases the expression of differentiated function genes in clonal cells in culture, in both human and bovine adrenocortical cells. Thus T antigen does not appear to act as an oncoprotein. Rather than being tumorigenic, it acts to increase differentiated gene expression and may therefore permit better cell function. Further research is necessary to clarify these questions.

In a set of planned experiments, genetic modification will be used to create a bovine DHEA-secreting cell and we will study the function of this tissue in the mouse. This was proposed in the grant application. However, due to the technical difficulties which had to be addressed, we are only now able to perform these experiments. They will be performed within the remaining period of this grant, as extended (to August 1999). Our previous research indicated that the key gene for the production of DHEA is 3β-hydroxysteroid dehydrogenase and that it is the low expression of this gene in the zona reticularis cell which

causes the cells to make DHEA. The absence of DHEA production in species like the cow and most non-human species results from the fact that the 3β-HSD level is much higher, throughout the adrenal cortex. We have made an antisense construct against bovine 3β-HSD. When introduced into bovine adrenal cells, this plasmid will be expected to lower 3β-HSD mRNA, protein, and activity. Therefore it will increase DHEA production. The reason for our confidence that lowering 3β-HSD activity will increase DHEA synthesis is that the same can be accomplished by chemical inhibitors of 3β-HSD, such as trilostane, in bovine adrenal cells. If this model is successful, it will not only show the effects of DHEA on the mouse, such as androgenization and the increased growth of androgen-dependent breast cancer cells, but also it will form a model of congenital adrenal hyperplasia. This will be useful for testing several concepts about regulation of adrenal growth in vivo. In congenital adrenal hyperplasia, the absence of the gene for one or more of the steroidogenic enzymes causes the cells to be unable to secrete the end-products, such as cortisol, and they secrete precursors. The precursors have adverse effects, such as androgenization in females. The absence of the end-product causes that the normal feedback to the hypothalamus and pituitary to be interrupted. ACTH is oversecreted and is thought to drive the increased growth of the adrenal gland. These effects may be seen in the mouse model; i.e., when 3β-HSD antisense cells are transplanted, the lowered production of cortisol would be expected to result in increased ACTH and increased growth of the adrenal tissue. This model would be useful beyond the study of DHEA in the mouse because it would show that regulation of adrenal size and proliferation can be modeled in the *scid* mouse/adrenocortical cell transplant system.

3. A third major area we have studied under this grant is the nature of the transcription factors which regulate the 3β-HSD gene and how its level of expression is suppressed in the zona reticularis. We began this work by observing that the differences between the type I and type II human 3β-HSD genes are slight. They are very similar in their promoter and regulatory elements, yet the type I gene is expressed in most tissues throughout the body and the type II gene is expressed only in steroidogenic tissues; specifically, within the adrenal cortex, only in the zona glomerulosa and the zona fasciculata. In examining the differences between the genes, one key area in the first intron came to our attention. We decided to examine if there are proteins in the adrenal cortex which bind specifically to this sequence. Our work, as summarized in previous reports, resulted in the identification of a DNA-binding protein present in the human zona fasciculata, but not in the zona reticularis, and also present at high concentrations in the bovine adrenal cortex. This was shown by gelshift assay and Southwestern blotting. We are currently part way through the process of purifying this protein from the bovine adrenal cortex in order to identify it and then to study its expression in the zones of the human adrenal cortex. In the first steps of the process the protein has been separated on a DEAE column with a ~30-fold enrichment as judged by gelshift assay. In a second step, the protein is being purified by binding to a biotinylated oligonucleotide attached to streptavidin magnetic beads. We anticipate that this work will be completed within the next few months and that following microsequencing of the protein, we will be able to commence studies on expression of this gene and its relevance for the differentiation of the adrenal cortex. If it is, in fact, a transcriptional regulatory protein that is differentially expressed between the zones, it will be the first example of such a protein. No transcription factors which differ in levels between the zones and thus regulate the relative production of mineralocorticoids, glucocorticoids, and androgens have yet been identified.

4. Growth of androgen-dependent MCF-7 tumors in *scid* mice. To test the effect of human adrenal androgens on growth of estrogen-responsive breast cancer, we have developed growth of human breast cancer in *scid* mice in an androgen-dependent manner. MCF-7 cells, a well-studied human breast cancer cell line, were transfected with aromatase cDNA, thus mimicking the normal situation of the human breast, which is exposed to estrogens derived

from circulating androgens via stromal tissue aromatase. We showed that these cells can grow as an androgen-dependent transplantable tumor line. Future studies will be designed to assess the effect of androgens secreted by transplanted adrenal cells on tumor growth.

Conclusions

The *scid* mouse/human adrenocortical tissue model has been set up and has been shown to be useful for studying human adrenal physiology, cell biology, and molecular biology. The development of clonal adrenocortical tissues in the *scid* mouse, which can also be genetically modified, has created the possibility of using genetically modified bovine adrenocortical cells as a model for adrenal androgen-producing cells in the mouse. Progress in identifying transcription factors regulating the key enzyme of adrenal androgen biosynthesis, 3β-hydroxysteroid dehydrogenase, will allow the study of adrenocortical cell differentiation and zonation, and thus the basis for androgen biosynthesis by the human adrenal cortex. The development of MCF-7 cells as an androgen-sensitive transplantable tumor line will allow the action of human adrenal androgens secreted by adrenocortical tissue in the *scid* mouse on human breast cancer growth.

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